1919
L57

Levinson: and Fisch

Study of coagulation in embryonic blood.

THE UNIVERSITY
OF ILLINOIS
LIBRARY

1919
L59

A STUDY OF COAGULATION IN EMBRYONIC BLOOD

BY

SAMUEL AZOR LEVINSON
B. S. University of Illinois, 1917.
M. D. University of Illinois, 1919.

AND

MAX ELEAZER FISCH
B. S. University of Illinois, 1917.
M. D. University of Illinois, 1919.

W42
1919
L42

THESIS

Submitted in Partial Fulfillment of the Requirements for the

Degree of

MASTER OF SCIENCE

IN ANATOMY

IN

THE GRADUATE SCHOOL

OF THE

UNIVERSITY OF ILLINOIS

1919

Digitized by the Internet Archive
in 2014

http://archive.org/details/studyofcoagulati00levi

1919
L57

UNIVERSITY OF ILLINOIS

THE GRADUATE SCHOOL

June 23 1919

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Samuel A. Levinson

ENTITLED A Study of Coagulation in Embryonic Blood

BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

V. E. Emmel
In Charge of Thesis

A. C. Eycleshymer
Head of Department

Recommendation concurred in*

Committee on Final Examination*

*Required for doctor's degree but not for master's

Mar 20 - C.L.
100
417534

1919
L57

A STUDY OF COAGULATION IN EMBRYONIC BLOOD

by

Samuel A. Levinson, B.S.,M.D., and Max E. Fisch, B.S.,M.D.

CONTENTS.

THE PAPER BOOK of
UIUC
MMXVIII

Introduction.

In the course of an experimental study of the origin of non-nucleated erythrocytes (Emmel[1]) certain observations were made indicating a slower coagulation time together with other striking differences in the reaction of embryonic blood as compared with that of the adult. The present work represents a more extended study of this subject with the purpose of ascertaining: first, the facts regarding the coagulation time of embryonic blood; second, to what extent the factors essential to coagulation are comparable in embryo and adult; and third, whether any of the conditions found in the embryonic blood may be of possible significance with reference to certain instances of abnormal coagulation in the adult.

With reference to the literature on this subject, it appears that, with one exception, (Boll[2], 1870), so far as we have been able to ascertain, there is no data recorded regarding coagulation in embryonic blood.

II. Coagulation Process in Normal Embryonic Blood.

1. Material and Technique.

The present study of coagulation is largely confined to pig embryos of 100 to 270 mm. in length. Through the courtesy of Swift and Company, this material could be obtained from the uterus under very favorable circumstances within a short interval after the killing of the parent animal. Only embryos in apparently perfectly normal condition were used in which the heart was still beating or would, in any event, respond to stimulation --- a point of considerable importance, since it was found that in the blood from embryos in which the heart was not beating the coagulation time was materially reduced.

In each case the umbilical cord was ligated and cut and the embryo removed from the uterus. Part of the anterior thoracic wall was then cut away, the pericardial sac opened, and the heart exposed. The needle (platinum) of a graduated hypodermic syringe was immediately inserted into the left ventricle and special care taken to obtain the blood without the inclusion of air bubbles or tissue juices. This

blood was then transferred in 0.5 cc. quantities to a series of test tubes, again exercising equal care against the introduction of air.

In general the technique used is in accordance with that of Lee and White[3]. Test tubes of varius sizes were tried but the best results were obtained with tubes having an internal diameter of 9 mm. and a length of about 9-10 cm. The glassware and syringes, thoroughly cleaned in chromic acid, were rinsed with physiological salt solution previous to use in each experiment. The coagulation of the blood was determined by tilting the test tube at short intervals and noting the formation of a sliding clot or a solid gel sufficient in the latter case to withstand the complete inversion of the tube. These experiments were made at a room temperature of about 21 C.

2. Coagulation Time and Character of Clot.

The following table presents the data for the coagulation time of the blood obtained from 18 embryos of various sizes. These results are typical of some fifty different experiments

Table I. Normal Coagulation Time for Embryonic Blood.

Size of embryos in mm.	Volume of blood in cc.	Coagulation time in minutes.	
		First evidence of fibrin observed.	Formation of Clot.
I00	0.5	25	30
I20	0.5	I9	28
I30	0.5	24	30
I35	0.5	I8	30
I40	0.5	I5	2I
I50	0.5	I2	22
I60	0.5	I2	20
I70	0.5	I2	2I
I80	0.5	I6	20
I90	0.5	I7	23
200	0.5	I9	22
2I0	0.5	I2	2I
220	0.5	I2	24
240	0.5	I8	2I
240	0.5	I7	2I
250	0.5	I7	24
250	0.5	I7	2I
270	0.5	I2	20
	Average Coagulation Time	I6.3 min.	23.3 min.

In these data it will be observed that the average normal coagulation time for the blood of these embryos is 23.3 minutes.

This is in marked contrast to the following results obtained for the blood of the adult pig. This blood had been allowed to flow into a clean receptacle as it escaped from the cut carotid and jugular vessels of the stabbed animal and its coagulation time determined for oxalated and non-oxalated blood by the same technique as previously described for the embryo.

Table II. Normal Coagulation Time for Adult Blood.

Non-oxalated		Oxalated		
Volume of blood in cc.	Coagulation time in minutes.	Volume of blood in cc.	Calcium chloride 0.5% in drops.	Coagulation time in minutes.
0.5	3	0.5	I	4
0.5	3	0.5	2	4
0.5	4	0.5	3	3
0.5	3	0.5	3	4

On the basis of this data it is evident that the coagulation time for embryonic blood is greatly delayed as compared with that of adult blood, --- the coagulation time for the embryo being nearly six to eight times greater than that of the adult.

With reference to these results certain points are to be noted regarding the process of coagulation as observed in these experiments on embryonic blood. The normal clot never attains the density nor firmness comparable to that of the adult blood. As a rule the clot is in the nature of a mass of semi-solid gel, which at its maximum is usually equal in volume to about two-thirds the total quantity of blood. Since this mass or lump may be made to move back and forth by tilting the tube, it may be appropriately described as a sliding clot. In contrast to the adult condition this clot is seldom of such a character as to engage the entire volume of blood to a degree sufficient to permit a complete inversion of the tube. Not infrequently the sliding clot is very small if not entirely absent.

The first indication of coagulation to be observed consists in the appearance of small particles or clumps of fibrin which are almost invariably deposited at the side of the tube. The formation of a complete gel or clot occurs within an average of about 7 minutes after this first appearance of fibrin as shown in Table I. Within a short time (30-50 minutes) this gel or clot retracts into a small compact mass as shown in figure I. It is of interest to note that, if the blood is not too greatly disturbed, the initial deposit of fibrin appears to form a nucleus around which the entire mass of fibrin accumulates during clot retraction. In some instances it appears that the total quantity of fibrin may be deposited in a compact mass without the formation of an intermediate gel-stage and thus account for the occasional absence of a sliding clot.

With refernece to the age of the embryos the data, in general, indicates some reduction in coagulation time in the oldest embryos, although it appears that the differences are not pronounced within the periods of development to which the present study is confined. In this connection it is to be

taken into account that size measurements alone can not always be taken as an absolute index to the age and degree of embryonic development, since embryos taken from the same uterus may differ by as much as 30 to 40 mm. in length.

III. Analysis of Possible Factors Involved in the Greater Coagulation Time of Embryonic Blood.

On the basis of our present understanding, the coagulation of blood in general is primarily dependent upon the interaction of three factors, prothrombin, calcium, and fibrinogen. Since the coagulation of embryonic blood is, as has just been demonstrated, much slower than that of the adult the question naturally arises whether these factors are equally active in the the embryo or whether other conditions are to be taken into account. The following analysis has been made with the view of attaining data toward the solution of this problem.

1. Blood Platelets.

a. Platelet Count.

Of these three fundamental factors of coagulation, one of them, the prothrombin, is primarily a derivative of blood

platelets. In many cases of pathological hemorrhage, such as hemophilia and purpura hemorrhagica, the abnormal variations in coagulation are found to be primarily related to quantitative or qualitative deficiencies in this platelet material. In view of this fact our attention was first directed to the subject of the platelet content of the embryonic blood.

From an examination of properly stained preparations of the blood from pig embryos it is obvious that platelets are present in great numbers. Jordan[4](p.404) has recently shown that blood platelets are very abundant in even 12 mm. pig embryos. For our present purpose, however, the crucial point involves a quantitative estimation of these elements based upon numerical data. Such data is presented in Table III.

Table III. Platelet Count for Embryonic and Adult Blood.

Size of embryo in mm.	Average number per cmm.
25	340,000
40	448,000
45	296,000
95	415,000
120	464,000
140	422,000
270	800,000
Adult	588,000*

* Derived from a number of counts in which platelet content varied from 544,000 to 932,000.

In making the platelet count 3% sodium citrate was used in accordance with the method described by Ottenberg and Rosenthal.[5] The above averages are based upon three or more counts in each case. The platelet counts show a surprising number of platelets in the embryonic blood. With the exception of certain instances as in the case of 270 mm. embryo, the platelet count is apparently somewhat less than that of the adult. This

difference, however, does not appear sufficient to account for the delay in the coagulation of embryonic blood. In the case of the human blood, with an average of about 250,000 platelets per cmm., pathological hemorrhage as shown by Minot[6] (p.1105) was not found to occur until the platelet count fell below 60,000. This represents a drop of 75% in man, whereas in no instances in pig ambryos between 100 and 270 mm. did the platelet count fall below 27% of the average for the adult.

b. Coagulation Reaction Upon the Addition of Platelet Preparation from Adult Blood.

In attempting to determine to what extent blood platelets may be involved in abnormal coagulation of blood, it is evident that a quantitative determination of the platelet content is not necessarily sufficient, as in the case of hemophilia, for example, where the platelets may be present in approximately normal number but still be abnormal in their activity (Lee and Minot[7] p. 80). In view of this possibility the following experiments have been made in which preparations of platelet material from adult pig blood were added to embryonic blood with the results

given in Table IV.

Table IV. Effect of Addition of Adult Platelet Material on Coagulation of Embryonic Blood.

Size of embryo in mm.	Volume of blood in cc.	Coagulation time in minutes.	
		Normal control.	Blood plus 2 drops of platelet suspension.
I00	0.5	30	I0
I30	0.5	30	5
I40	0.5	2I	7
I60	0.5	20	I3
I80	0.5	20	4
200	0.5	22	I0
220	0.5	24	8
240	0.5	2I	II
250	0.5	2I	I2
270	0.5	20	4
	Average Coagulation Time	23 min.	8.4 min.

The suspension of platelet material used in our experiments was prepared in accordance with the technique of LeSourd and Pagniez.[8]

The blood, caught in clean receptacles as it escaped from the vessels of the stabbed animal, was immediately transferred to 50 cc. centrifuge tubes and mixed with equal volumes of a solution of 0.4% sodium oxalate in physiological saline, the final mixture of blood with this solution containing 0.2% sodium oxalate. These tubes were then centrifuged at 1500 revolutions per minute for 10-15 minutes and a little more than the upper half of the clear plasma pipetted off and again centrifuged at 1500 revolutions for 15 minutes longer to throw down any remaining cells. This oxalated plasma was then centrifuged in 15 cc. tubes for one half hour at 3000 revolutions per minute. The clear supernatant plasma was then decanted leaving a small quantity of sticky grayish white mass of platelet material at the bottom of the tube. A quantity of a solution of 0.2% sodium oxalate in physiological saline was then added to each tube, the platelets stirred up in this fluid and the resulting suspensions for four tubes collected into one tube and again centrifuged for 15 minutes at 3000 revolutions. The supernatant fluid was poured off and the platelets from the entire volume of blood

(200 cc.) extracted with 2 cc. of distilled water. The resultant milky white suspension of platelets was used for experimental purposes within 24 hours after preparation.

These data demonstrate very striking results upon the addition of platelet suspension. The coagulation time of 23 minutes for the normal controls has been reduced to 8.4 minutes by the addition of platelet material --- a reduction of nearly 75%. The process of coagulation was also attended by the formation of a larger and more homogeneous clot or gel. Not only are these results of interest in that they show that the coagulation time can be materially shortened, but they seemed for the moment to emphasize some abnormal activity of the blood platelets or prothrombin as a factor in the greater coagulation time of embryonic blood. But as will develop later it was found that this conclusion could not be maintained.

Upon examination of this data it becomes obvius that the effect of the addition of platelet material is by no means constant. In some cases the blood coagulated in 12-13 minutes, whereas in other instances the clot was formed in 4 minutes

---- a period equivalent to that of the adult coagulation time. No doubt this variation may be due, in part, to qualitative differences in the platelet preparation, for the suspensions differed in opacities, and, as emphasized by Lee and Vincent[9] (p. 407), it is difficult to obtain platelet suspensions of constant strength. With reference to this point we did not feel convinced, however, that platelet material alone was sufficient to account for the marked degree of variation in results obtained. This raised the question as to whether other factors may not be involved in our problem. That these results can not be due to mere dilution of the blood was shown conclusively by tests with physiological salt solution and distilled water in which the results were negative.

2. Effect of Addition of Calcium on Coagulation of Embryonic Blood.

In view of the preceding considerations with reference to the platelet experiments, attention was next directed to the subject of calcium. Table V presents the results of a series of experiments showing the effect upon the addition of 2 drops

of 0.5% calcium chloride to embryonic blood.

Table V. Effect of Addition of Calcium Upon Coagulation Time of Embryonic Blood.

Size of embryo in mm.	Volume of blood in cc.	Coagulation time in minutes.	
		Normal control	Blood + 2 drops of 0.5% $CaCl_2$
I00	0.5	30	I3
I20	0.5	28	I0
I30	0.5	30	I3
I35	0.5	30	II
I40	0.5	2I	II
I50	0.5	22	8
I60	0.5	20	8
I70	0.5	2I	9
I80	0.5	20	II
I90	0.5	23	I2
200	0.5	22	II
2I0	0.5	2I	9
220	0.5	24	9
240	0.5	2I	I2
250	0.5	2I	8
270	0.5	20	9
	Average Coagulation Time	23.3 min.	I0.3 min.

Here again, much to our surprise, we get a reduction of over 50%. This is not quite as pronounced as in the case of platelet material, but the results are more uniform and in any event show that the addition of calcium has a decided effect on coagulation process. These experiments in themselves would seem to indicate a deficiency of calcium in the embryonic blood --- a deficiency which would of course seriously interfere with coagulative processes. This conclusion, however, is contradicted by the results obtained in the platelet experiments. It is generally agreed that free calcium ions constitute a specific element essential in all coagulation of blood. Since the addition of platelets materially facilitate coagulation, it appears evident that under these experimental conditions there must have been sufficient amount of calcium ions available for coagulation.

These results consequently present a dilemma since on the one hand the platelet experiments alone would seem to indicate a deficiency on the part of prothrombin rather than calcium, whereas, in the calcium experiments the situation appears re-

versed indicating a deficiency in the calcium rather than prothrombin.

3. Reaction of Embryonic Blood to Tissue Juice.

Directing attention to one of the two horns of the preceding dilemma, i.e. the reaction with platelet material, it will be observed that platelets were used in the form of a suspension, and the question arises whether the effect of the suspension on coagulation may not be due to elements other than its prothrombin content. In this connection it is to be recognized that in addition to prothrombin, blood platelets contain a second substance, thromboplastin, (Bayne-Jones[19]), corresponding in its reaction with the phosphatide designated as cephalin (Howell[11], p. 290).

In the preparation of the platelet material used in our experiments, the technique was such that the thromboplastin is still retained in the suspension, and it is therefore possible that this substance constitutes an important factor in the preceding results. To determine whether this is the case experiments were made testing the effect of tissue extract on coag-

ulation, since it has been shown that this active substance is present in such extract (Howell", p. 290).

The extravts were made of tissues of the heart and umbilical cord of about 200 mm. pig embryos. These tissues were rinsed in water to remove any excess of blood and extracts made in both saline and water by grinding in a mortar with sand. This mixture was then transferred to a 15 cc. centrifuge tube, centrifuged for 5 minutes, and the supernatant fluid removed. This fluid or extract was then used in a series of experiments with the results shown in Table VI.

Table VI. Effect of Tissue Extracts Upon Coagulation Time of Embryonic Blood.

Size of embryo in mm.	Volume of blood in cc.	Coagulation time in minutes.		
		Normal control	Blood + 2 drops heart extract.	Blood + 2 drops cord extract.
I00	0.5	20	4	4
I40	0.5	I9	4	4
I70	0.5	I8	3	3.5
200	0.5	I3	3	4
230	0.5	I8	4	4
250	0.5	I5	2	3
	Average Coagulation Time	I7.2 min.	3.3 min.	3.7 min.

On the basis of these results it is clearly demonstrated that tissue extracts have a decided effect on coagulation time. Not only is the time greatly reduced, but what appears even more remarkable is the fact that the coagulation time is brought down to the normal time for the adult blood, viz. 3-4 minutes. It appears obvious, therefore, that in spite of the fact that the embryonic blood normally shows a long coagulation time, it nevertheless becomes apparent that this blood not only contains all the constituent elements essential to coagulation, but that these elements will also react under proper conditions in a time equivalent to that of the adult blood.

These results were furhter verified by blood which had come in contact with cut tissue surfaces by merely being allowed to run over the cut surfaces of the heart and cord and found to coagulate in 3-4 minutes. It should be stated that in all experiments with tissue extract not only was the coagulation time thus reduced but the clot was of a much firmer character and in all cases permitted complete inversion of the test tube.

From the standpoint of the results with tissue extract alone it would appear that the delayed coagulation in embryonic blood must be due to some inhibitory factor. On the basis of our general understanding of the role of tissue extracts (Howell", p. 292) it might be assumed that this factor is solely in the nature of an antithrombin or antiprothrombin. This conclusion loses some of its force, however, in view of the results attained with calcium, the addition of which also brings about a reduction in coagulation time --- a result theoretically inconsistent with the preceding assumption, since this involves the neutralization of antithrombin or antiprothrombin by calcium.

These facts indicate that in attempting to determine the nature of the inhibitory factor or factors in embryonic blood, consideration must be given to their possible interaction with calcium as well as with prothrombin.

4. Calcium Content of Embryonic Blood.

In connection with the second aspect of the preceding dilemma it became of importance to ascertain, if possible, the

facts regarding the calcium content of embryonic blood. To this end a chemical analysis of adult and embryonic blood was undertaken by Dr. P. G. Albrecht of the Department of Physiological Chemistry who kindly furnished us with the following data from his as yet unpublished results, which show that in place of a deficiency there is a pronounced excess of calcium present in embryonic blood as compared with the adult.

Table VII. Determination of Calcium (as Calcium) for Embryonic and Adult Blood.*

Quantity	Calcium in mgm.		Ratio of embryo to adult.
	Embryo (200mm.)	Adult	
Total blood 100 gms.	9.5	6.744	7:5
Plasma 100 cc.	11.73	8.51	7:5

* The method used was that of Halverson-Bergeins[12] modification of McCrudden.

Since on the one hand the results of the preceding experiments upon the addition of calcium indicate a deficiency in this element, while on the other hand the present data show that calcium is quantitatively present in excess, the suggestion arises that the calcium in embryonic blood is present in some combined form rendering the blood deficient in the free calcium ions essential to coagulation.

5. Effect of Barium and Magnesium on Coagulation.

Since the calcium is therefore evidently present in embryonic blood in some combined form, it becomes of interest to ascertain whether possibly the addition of other salts, such as barium and magnesium, might liberate some of this calcium and bring about a change in coagulation time. With this end in view the following experiments were made with the chlorides of barium and magnesium in 0.5% strength.

Table VIII. Effect of Addition of Calcium, Barium, and Magnesium on Coagulation Time of Embryonic Blood.

Size of embryo in mm.	Volume of blood in cc.	Coagulation time in minutes.			
		Normal control	Addition of 2 drops of 0.5% $CaCl_2$	Addition of 2 drops of 0.5% $BaCl_2$	Addition of 2 drops of 0.5% $MgCl_2$
I35	0.5	25	II	I4	I8
I80	0.5	I5	6	8	8
230	0.5	I8	6	7	7
250	0.5	2I	8	8	I0
270	0.5	I5	9	I0	II
	Average Coagulation Time	I9 min.	8.5 min.	I0 min.	II.75 min.

As a control to determine that these results were due to a liberation of calcium and not to an interaction with some other elements, such as prothrombin for example, the same experiments were repeated on oxalated embryonic blood as shown in Table IX.

Table IX. Effect of Addition of Calcium, Barium, and Magnesium to Oxalated Embryonic Blood.

Size of embryo in mm.	Volume of blood in cc.	Coagulation time in minutes.			
		Normal control	Addition of 3 drops of 0.5% $CaCl_2$	Addition of 3 drops of 0.5% $BaCl_2$	Addition of 3 drops of 0.5% $MgCl_2$
I70	0.5	No clot	6	No clot	No clot
270	0.5	No clot	7	No clot	No clot

1. Added 2 more drops Ba. after ½ hour and no clot formed. Added 3 drops of Ca. after one hour and clot formed in 6 minutes.
2. Added 2 more drops Mg. after ½ hour and no clot formed. Added 3 drops of Ca. after one hour and clot formed in 7 minutes.
3. Two instead of 3 drops of calcium used.
4. Added 2 drops Ca. after 25 minutes, and clot formed in 5 min.
5. Same as number 4.

With reference to our present purpose these results are very significant. Barium and magnesium, although somewhat slower in reaction, bring about a reduction in coagulation time of non-oxalated embryonic blood in a manner comparable

to that of calcium as shown in Table VIII. These same elements in contrast to the action of calcium, are negative in reaction with the oxalated embryonic blood. On the basis of the specificity of calcium these results apparently permit of but one conclusion; namely, that the action of barium and magnesium is due to a liberation of an amount of calcium sufficient to permit the interaction essential for the process of coagulation to become operative --- thus furnishing confirmatory evidence for the conclusion that the calcium in embryonic blood is present in some combined form.

6. Fibrin and Fibrinogen.

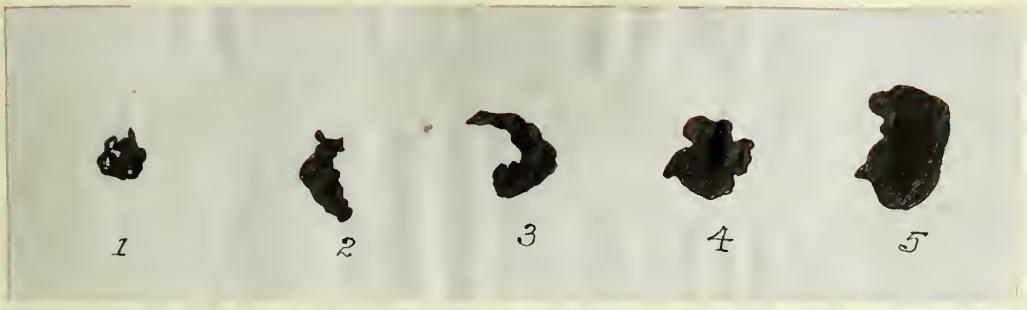
Reverting to fibrinogen, the third of the factors under consideration as essential to coagulation, the following observations were made. In the case of the other two factors it has been possible to show that in a comparison of embryonic and adult blood of the pig, the calcium and platelet content in the blood of 100-270 mm. embryos is more or less comparable to that of the adult. In the case of fibrin certain differences were observed in the course of the preceding ex-

periments. In the coagulation of normal embryonic blood to which calcium and platelets were added, it was observed that the clot remaining after complete retraction was somewhat larger in the case of platelet experiments than in the case of calcium. In both instances, however, the retracted clots were larger than the corresponding resultant clots in the case of the normal embryonic blood.

To compare the relative quantities of fibrin formed in embryo and adult, one cc. quantities of adult and embryonic blood were defibrinated by stirring with clean glass rods drawn out to narrow points. Tests were made with adult, normal embryonic blood, and normal embryonic blood to which calcium, platelets, and tissue extract had been added. The fibrin collected on the glass rods was freed from blood by washing in distilled water and kept in 5% formalin. Figures 1, 2, 3, 4 and 5 are drawings of typical cases showing relative quantities of fibrin obtained in embryonic and adult blood.

Figures 1,2,3,4, and5. Profile Projections to Show the Relative Size of the Masses of Fibrin Obtained from the Defibrination of 0.5 cc. Volumes of Embryonic and Adult Blood. The Embryonic Blood

was Taken from the Heart of a 200 mm. Embryo.



1. Normal embryonic control.
2. Embryonic blood plus 3 drops of calcium chloride.
3. Embryonic blood plus 3 drops of platelet suspension.
4. Embryonic blood plus 3 drops of tissue extract.
5. Adult pig blood.

While data of this character lack the precision of measurements by weight, they nevertheless appear to clearly demonstrate that there is a pronounced difference between embryo and adult. In the case of the embryo it may be observed that in the normal coagulation of embryonic blood only a very small amount of fibrin is formed, whereas more fibrin appears to be formed upon the addition of calcium and that the maximum amount is attained in the presence of platelet preparations and tissue extract. At its maximum the amount of fibrin formed in embryonic blood is less than 0.1 of that obtained from an equal volume of adult blood. If the quantity of fibrin can be taken as a fairly

reliable index of the relative amount of fibrinogen present, it appears that, in contrast to the comparative calcium and platelet content, the fibrinogen content of the embryonic blood is far below that of the adult. This difference in fibrin and fibrinogen content is in itself, evidently not primarily responsible for the greater coagulation time of embryonic blood, for it is obvious that in the presence of tissue extract, in spite of this smaller amount of fibrinogen, the coagulation time becomes equivalent to that of the adult.

7. Bile.

The preceding analysis demonstrates that the delay in coagulation of embryonic blood is primarily a result of some condition pertaining to calcium. Furthermore, it has also been shown that this is due not to a deficiency in the calcium content, but to some combination of the calcium rendering it unavailable for coagulation. This raises the question as to the factor which may be thus holding the calcium in check.

If we consider the various types of pathological hemorrhage in man, it is of interest to find that in the case of jaundice

or icterus we encounter conditions especially significant with reference to our present subject. In the case of jaundice, as has long been recognized, there is a marked delay in the coagulation time of the blood. In the history of the subject this delay has been assigned to various causes, but in the more recent literature, as demonstrated by Lee and Vincent[13] and stated by Wells,[14] there is a convergence of opinion toward the conclusion that "In icterus a notable tendency to hemorrhage seems to depend upon the binding effect of the calcium of the blood by the bile pigments" (Wells[14] p. 321). This conclusion becomes highly suggestive with reference to embryonic blood, for, it has recently been shown by Bang,[15] that in the case of the new born infant a notable amount of bile pigment is constantly present in blood obtained from the umbilical cord. This pigment shows a constant increase during the first few days and, if marked, the transition is readily made to the condition designated clinically as icterus neonatorum.

Yllpo[16] in his earlier article on new born and prematures attains similar results for man, but with exception of the

horse comparable conditions were not found in other mammals. In the case of such animals as wolf, deer, leopard, yak, dog, pig, in contrast to horse and man, only small quantities of bile pigment, if any, were found present in the blood of the new born at birth. In the discussion of his results, he concludes that toward the end of intrauterine life, in the case of man and horse, there is a notable quantity of bile constantly present in the circulating blood. In the case of puppies data is advanced in support of the theory that the same is true in the intrauterine life of other mammals; in the latter case, however, it is pointed out that in contrast to the conditions in the case of man and horse, the excess of bile in the circulation has largely disappeared at the time of birth, and that with respect to functional activity of the liver and excretion of bile, the young of the horse and man may therefore be regarded as prematurely born.

These reults upon the newly born consequently focus attention upon the possibility of the presence of bile in the blood of the pig embryos used in our present experiments and that this bile

may be a factor of primary importance affecting the process of coagulation. Toward this end a qualitatinve examination was made of serum obtained from blood of 100-270 mm. pig embryos. Tests were made in accordance with Huppert's technique in which the bile pigments are precipitated with milk of lime, liberated from the precipitate by boiling in strong acid-alcohol and its presence demonstrated by the appearance of a typical color reaction. In making the test 25 cc. serum were obtained from several embryos by centrifuging the blood and in order to secure the pigment in more concentrated form, the precipitate was rediluted to only 10-15 cc. in making the final color reaction test. The results furnished conclusive evidence of the presence of bile in blood of these embryos.

The presence of this bile seems to furnish a clue to the solution of our problem. As already indicated it has been shown that in the case of icterus (Lee and Vincent[13]) the presence of bile may interfere with the participation of calcium in coagulation, and the suggestion consequently arises that the same is true of the embryonic blood. Since as already demonstrated

in the preceding experiments, the coagulation time for embryonic blood could be greatly reduced by the addition of calcium, and even brought down to the coagulation time obtained for the adult by the addition of tissue extract, it became desirable to ascertain whether a similar experimental condition could be produced in the normal adult blood by the mere addition of bile.

To this end the following experiments were made in which given concentrations of bile obtained from gall bladders of adult pigs were added to definite quantities of adult pig blood. It was found that the amount and concentration of bile added to the blood is of primary importance in obtaining any given effect on the coagulation time. In order to approximate embryonic conditions the addition of bile should be such as to bring about a coagulation of the adult blood in a time comparable to that of the embryo, that is, in about 20-30 minutes. To ascertain the requisite amount of bile for this purpose, tests were made with various concentrations as shown in the following table. The bile for this purpose was removed from the gall-bladder, filtered and then diluted with distilled water to

give the various percentages of concentrations indicated.

Table X. Showing Effect of Different Concentrations of Bile Upon Coagulation Time of Adult Pig Blood.

Volume of blood in cc.	Concentrations of bile in percentages.	Drops of bile dilutions added	Coagulation time in minutes.
0.5	1	2	3
0.5	3	2	3
0.5	5	2	3
0.5	7	2	3
0.5	10	2	3
0.5	20	1	3
0.5	20	2	6
0.5	20	2	7
0.5	20	3	30
0.5	20	3	20
0.5	20	3	27
0.5	25	1	14
0.5	25	1	10
0.5	25	2	22
0.5	25	3	30
0.5	25	3	25
0.5	50	1	2 hours
0.5	100	1	No clot*

* Only loose strands of fibrin found.

On the basis of these experiments, it was determined that 3 drops of 20%-25% solution of bile constitutes the optimum amount necessary to give a coagulation time of about 20-30 minutes for the adult blood. The blood when thus treated contains 8%-10% bile in the final mixture.

Having determined the optimum amount of bile necessary for the present purpose the following experiments were made to test the coagulation reaction of adult blood to which bile had been added as compared with that of normal embryonic blood.

Table XI. Experiments Upon the Coagulation Time of Adult Pigs Blood in the Presence of Bile.

Volume of blood in cc.	Drops of 20% bile.	Coagulation time in minutes.		
		Control	Addition of 3 drops of 0.5% $CaCl_2$	Addition of 3 drops of tissue extract[1]
0.5	2	7	4	2.5
0.5	3	27	15	3
0.5	3	20	8	4
0.5	3[2]	25	8[3]	4
	Average Coagulation Time	19.7 min.	8.7 min.	3.4 min.

1. Extract was made from embryonic heart tissue.
2. In this case 25% bile was used.
3. This same blood was also tested with 0.5% $BaCl_2$ and 0.5% $MgCl_2$ (3 drops being used in each experiment) giving a coagulation time of 10 minutes in each case.

These results appear very significant. The coagulation time of the adult blood when delayed to about 26 minutes by the addition of bile can, by the addition of calcium, barium and magnesium be reduced to an average of 9.6 minutes and upon the addition of tissue extract be brought back to the normal time of 3-4 minutes. These results accord to a remarkable degree, point by point, with the results previously obtained for the embryo (Tables 8,9) and demonstrate that by the mere addition of bile conditions can be produced in the adult blood which appear essentially indentical to those of the embryonic blood.

Since in the course of the preceding analysis of the variuos factors involved in coagulation, no significant differences could be demonstrated between the blood of the embryo and that of the adult, other than the presence of bile in the case of the former, the present data, by exclusion, consequently justify the following conclusion: namely, that it is the bile content found in the circulation of 100-270 mm. pig embryos that constitute the primary factor accounting for the greater coagulation time encountered in the blood of these embryos.

IV Discussion.

In discussing certain aspects of the preceding work, attention may be directed to the following points:

1. When, in the earlier phases of the present study, it was found that the greater coagulation time of embryonic blood could not be due to a quantitative deficiency in blood platelets as compared with adult, the suggestion arose that possibly the conditions here might be comparable to that of hemophilia, for, as seems well established, in the case of hemophilia the platelet content, numerically, is also essentially equivalent to that of normal blood (Lee and Minot[7], p. 80). Subsequent data, however, has shown that such a comparison can not be made. On the contrary in the place of the condition in the embryonic blood being comparable to hemophilia, it has been found to be more nearly that of icterus in which the bile constitutes the primary factor in the abnormal coagulation time.

2. Since it appears that the inhibitory action of bile is due to a large degree to the combination of its pigment with calcium (Wells[14]), the question arises as to how the process

of coagulation is initiated in normal embryonic blood by the addition of tissue extract. In the case of calcium experiments the results are obviously due primarily to the introduction of calcium ions in excess of the amount which enters into chemical combination with the bile present. In the case of the tissue extract it appears obvious that the initiation of coagulation must have involved an interaction with the constituents of bile of such a character as to liberate the necessary amount of calcium. Such an interaction seems clearly demonstrated in the preceding experiments with adult blood to which bile had been added (Table XI). This is in contrast with the results of Lee and Vincent[13] who, in their study of obstuctive jaundice, state that they "attempted to determine whether the action of bile could be neutralized by cytozyme or serozyme, but no results were obtained" (p. 63).

On the basis of this conclusion the suggestion arises that the normal coagulation of embryonic blood, so far as bile is concerned, involves a process comparable to that of the addition of tissue extract or cephalin, only on a smaller

scale. In the embryonic blood, in vitro, through the general disintegration of cellular elements, a certain amount of tissue substance, cephalin, is slowly set free in the plasma. This tissue substance neutralizes the bile and ultimately liberates a sufficient amount of calcium to bring about coagulation.

Of interest in this connection is the fact that the first formation of fibrin takes place almost invariably at the side of the tube (p. 8). This fibrin is situated at a level between the red cells and supernatant plasma. Evidently this is due to the disintegration of the white cells and platelets which tend to accumulate at this level. These cellular elements coming in contact with the sides of the tube liberate cephalin and this in turn initiates the formation of fibrin at this location.

3. The fact that there is a pronounced difference in the quantity of fibrinogen in the blood of the embryo as compared to that of the adult, and that under proper conditions the coagulation time becomes the same for both embryo and adult, demonstrates that the quantity of fibrinogen present in the blood

does not play an important role in determining the coagulation time of blood -- a conclusion confirmatory of Whipple's[17] observation (p. 390) in certain cases of hepatic cirrhosis and purpura.

While the amount of fibrinogen is, therefore, of minor importance so far as coagulation time is concerned, it is to be noted that the actual quantity of fibrin formed in the embryonic blood was greater upon the addition of tissue extract than in the case of the experiments with calcium. In a recent study of the effect of bile on the clotting time of blood Haessler and Stebbins[18] conclude that bile, through its bile salts, interferes with conversion of fibrinogen into fibrin. It is consequently possible that the greater amount of fibrin obtained in the case of tissue extract may be due to the calcium interacting primarily with bile pigments, whereas the action of tissue extract involves not merely the pigments but also the bile salts, and thus account for the increased amount of fibrin.

V. Summary.

The results of the present study of coagulation in embryonic blood can be summarized as follows:

Coagulation Time and Character of Clot in Embryonic Blood.

1. In pig embryos of 100-270 mm. the average coagulation time of the blood was found to be 23 minutes. This represents a coagulation time for the embryo 6-8 times greater than that obtained for the adult.

2. The first evidence of coagulation consisted in the appearance of small masses of fibrin deposited almost invariably at the side of the test tube. The ensuing coagulum is as a rule in the nature of a sliding clot, never attaining any marked degree of density or firmness.

Analysis of Possible Factors Involved in the Greater Coagulation Time of Embryonic Blood.

3. Numerically the blood platelets varied from 415,000 - 800,000 per cmm. a content not differing in any significant degree from that of the adult in which the average was found to be about 588,000 with a variation from 544,000 - 932,000.

4. By the addition of platelet material obtained from the adult pigs blood the average coagulation time for the embryonic blood was reduced to an average of 8.4 or 75%.

5. Upon the addition of 2 drops of 0.5% calcium chloride the coagulation time for embryonic blood was reduced to an average of 10.3 minutes -- a reduction of over 50%.

6. By the addition of tissue extract the embryonic coagulation time was brought down to an average of 3.7 minutes, a time essentially equivalent to that of the adult blood. The clot was of a much firmer character than obtained in either the normal coagulation or in the calcium experiments.

7. A chemical analysis demonstrated a calcium content in embryonic blood in excess of that of the adult, in the proproportion of 7:5.

8. Barium and magnesium, although somewhat slower in reaction, bring about a reduction in coagulation time of non-oxalated embryonic blood in a manner comparable to that of calcium. When added to oxalated embryonic blood, the same elements were negative in their action, thus furnishing confirm-

atory evidence that the calcium in embryonic blood is present in some combined form.

9. The fact that the maximum amount of fibrin obtained by defibrinating embryonic blood is less than 0.1 that obtained from an equal volume of adult blood, indicates a low fibrinogen content in the case of the embryo. Since, however, upon the addition of tissue extract the coagulation time of embryonic blood becomes equivalent to that of the adult, it appears that differences in fibrinogen content do not play an important role in determining coagulation time.

10. The presence of bile was demonstrated in the circulating blood of these embryos.

11. In a series of experiments it was also demonstrated that by the mere addition of certain percentages of bile to adult blood a condition could be produced in this blood essentially identical with that of the embryonic blood.

12. In the course of the preceding analysis of the various factors involved in coagulation, no significant differences could be demonstrated between the blood of the embryo and that

of the adult, other than the presence of bile in the case of the former, the present data, by exclusion, consequently justify the following conclusion: namely, that it is the bile content found in the circulation of 100-270 mm. pig embryos that constitutes the primary factor accounting for the greater coagulation time encountered in the blood of these embryos. This fact also indicates a condition in embryonic blood comparable to that of icterus.

13. In the case of the calcium experiments the results are obviously due to primarily to the introduction of calcium ions in excess of the amount which enters into chemical combination with the bile present. In the case of the tissue extract it appears that the initiation of the coagulation must have involved an interaction, with the constituents of bile of such a character as to liberate the amount of calcium essential to this purpose.

14. On the basis of the present data, the suggestion arises that the normal coagulation of embryonic blood, so far as bile is concerned, involves a process comparable to that of

the addition of tissue extract or cephalin only on a smaller scale. In the embryonic blood, in vitro, through the gradual disintegration of cellular elements, a certain amount of tissue substance (cephalin) is slowly set free in the plasma. This tissue substance neutralizes the bile and ultimately liberates a sufficient amount of calcium to bring about coagulation.

VI Literature Cited.

1. Emmel, V.E.: Concerning certain cytological characteristics of the erythrocytes in the pig embryo, and the origin of non-nucleated erythrocytes by a processs of cytoplasmic constriction, Amer. Jour. of Anat. 1914, Vol. 16, No.2.

2. Boll, F.: Ein beitrag zur kenntness der blutgerinnung, Archiv. fur Anatomie and Physiologie, 1870.

3. Lee, R.I. and White, P.D.: A clinical study of coagulation time of blood, Amer. Jour. Med. Sciences, 1913, Vol.145, P. 495.

4. Jordan, H.E.: The histogenesis of blood platelets in the yolk sac of the pig embryo, Anat. Record, 1919, Vol. 15, No. 7.

5. Ottemberg, R. and Rosenthal, N.: New and simple method for counting platelets, Jour. A.M.A. 1917, Vol. 69, P. 999.

6. Minot, G.R.: Pathological hemorrhage, Medical Clinics of North America, 1917-1918, Vol. 1, P. 1103.

7. Lee, R.I. and Minot, G.R.: The significance of blood platelets, Cleveland Medical Jour. 1917, Vol. 16, P. 65.

8. LeSourd, L. and Pagniez, P.: Le retraction du caillot sanguin et les hematoblastis, Jour. Phys. et Path. General. 1907, Vol. 9, P. 582.

10. Bayne-Jones, S.: The presence of prothrombin and thromboplastin in the bloodplatelets, Amer. Jour. Phys. 1912, Vol. 30, P. 74

11. Howell, W.H.: Coagulation of blood, Harvey lectures, 1916-1917, Series 2.

12. Halverson-Bergen.: Jour. Biolog. Chemistry, Vol.159, P. 159.

13. Lee, R.I. and Vincent, B.: The relation of calcium to the delayed coagulation in the blood in obstructive jaundice, Archiv. Inter. Med., 1915, Vol. 16, P. 59.

14. Wells, H.G.: Chemical pathology, 3d edition, 1918, P. 321.

15. Bang, F.: Recherches sur l'origine de l'icter des nouveaunes, Arch. mens. d'Obst. et Gynec., 1915, Vol. 8, P. 296.

16. Ylppo, A.: Icterus neonatorum (incl. I. n. gravis) und gallenfarbstoffsekretion beim foetus und neugeboren, Ztschr. f. Kinder., Orig., 1913.

17. Whipple, G.H.: Hemorrhagic Disease - Septacemia, Malena Neonatorum, and Hepatic Cirrhosis, Arch. Int. Med., 1912, Vol. 9, P. 365.

18. Haessler, H. and Stebbins, M.E.: Effect of Bile on the Clotting Time of Blood, Jour. Exp. Med., 1919, Vol. 29, No. 5, P. 445.

UNIVERSITY OF ILLINOIS-URBANA
3 0112 079828510